

SALIVARY CYSTATIN C- A DYNAMIC BIOMARKER OF PERIODONTAL DISEASE

ADITISHRIKRISHNADHAGE¹ & SAVITHA A. N²

¹MDS, Private Practitioner & Consulting Periodontist & Implantologist, Mumbai, Maharashtra, India

²MDS, Professor, Department of Periodontics, The Oxford Dental College, Hospital & Research Centre,
Bangalore, Karnataka, India

ABSTRACT

Background: The role of saliva in periodontal health is of great interest due to the presence of wide array of bioactive molecules offering protection against enzymatic tissue degradation seen in periodontal disease. Cystatin C is one such physiologically active antimicrobial protein well recognized due to its inhibitory potential against lysosomal enzymes, which are major contributors of periodontal disease. Aims and objectives: To assess severity of periodontal disease based on salivary cystatin C levels. Material and methods: Salivary cystatin C levels were evaluated and compared in periodontal health and patients with chronic periodontitis- smokers, chronic periodontitis non-smokers and aggressive periodontitis with 15 patients in each group.

The salivary samples were collected from each subject prior to the therapy and were analyzed for the levels of cystatin C by using the Quantitative Sandwich Enzyme Immunoassay technique. Results: Salivary cystatin C levels were highest in periodontal health when compared to periodontitis patients. Although, salivary cystatin C levels were slightly greater in smokers when compared to non-smokers with chronic periodontitis, the difference was not statistically significant. A positive correlation was observed between salivary cystatin C levels and gingival index & bleeding index. Aggressive periodontitis patients exhibited remarkably low levels of salivary cystatin C and a negative correlation with all the clinical parameters. Conclusion: These findings indicated that salivary cystatin C is a fairly significant biomarker of inflammation to assess periodontal disease severity, particularly in aggressive periodontitis patients.

KEYWORDS: Saliva, Biomarker, Cystatin C, Smoking, Periodontal Disease

INTRODUCTION

Periodontitis is a multifactorial infectious disease characterized by gingival inflammation and attachment loss in a susceptible host caused by a group of specific micro-organisms. Several biochemical events that occur during inflammation lead to the release of various enzymes by plaque bacteria and host lysosomal cysteine proteinases like cathepsin B, H, L and K, which are considered to be major contributors of periodontal tissue breakdown. Regulation of these host and microbial proteinases is an important biochemical aspect in the progression of periodontitis.¹

Several conventional methods have been followed over the years to assess severity of the disease and identification of probable individuals, who could be at a risk of periodontal breakdown in future. These include clinical parameters like bleeding on probing, probing pocket depth, clinical attachment level, radiographic assessment of alveolar bone loss, microbial and GCF analysis. These measures can only provide information about disease activity and severity, but they cannot predict future periodontal breakdown.²

More recently, genetic analysis has been in spotlight of the present day research, but the high levels of specificity, sensitivity, complexity and high cost depicts its limitations.³ Thus, the identification of patients at a risk of active disease represents challenge to both clinical investigator as well as the clinician.⁴ Presently, saliva is one such area of research which holds tremendous value in diagnostic medicine. Saliva is the defender of oral cavity and is of supreme importance in the maintenance of health and integrity of oral tissues.⁵ It can be easily and non-invasively collected. It contains an array of locally and systemically derived, physically active and functionally versatile markers of periodontal disease and hence it offers the basis for patient specific diagnostic tests for periodontitis.⁶ However, its diagnostic applications have several limitations. Its' physical and chemical nature is most of the times influenced by various drugs, systemic diseases as well as local factors such as contamination with food debris, shed cells and particulate matter. Also, most of the compositional studies of saliva are affected by proportionals of secretion from different salivary glands.⁷

Various endogenous proteinase inhibitors such as histatin, mucins, lactoferrin, lysozyme and the inhibitors of cysteine proteinases like cystatins are widely distributed in human saliva as well as several body fluids.⁸ Cystatin C belong to the class of superfamily of cysteine proteinase inhibitors. It is a non-glycosylated, basic, low-molecular weight protein which is highly expressed in secretory fluids including human saliva.⁹ It regulates inflammatory periodontal disease by inhibiting collagen degrading cathepsins and plays a pivotal role in tissue remodeling by down regulating protease activity.¹⁰ Several systemic conditions like nephropathy, diabetes mellitus, atherosclerosis, chronic liver cirrhosis etc. as well as environmental risk factors for periodontal disease such as smoking, have also been shown in association with an altered salivary cystatin C levels.⁹ Thus, it may serve as a useful biochemical marker for assessing periodontal disease status.

In light of above data, the present study is designed to gain insight into the contribution of actual levels of salivary cystatin C in periodontal health and its variability in different periodontal disease conditions such as chronic and aggressive periodontitis. It also evaluates the effect of environmental risk factor-smoking on the levels of salivary cystatin C. Hence, it emphasizes the potential use of salivary cystatin C as an important biomarker in periodontal research and could open up a new avenue in Periosteutics for designing a synthetic agent acting as an inhibitor of cathepsins that cannot be degraded in the proteolytic environment associated with periodontal disease.¹¹

MATERIALS AND METHODS

Total 60 systemically healthy male and female subjects aged between 15-55 years, without prior history of antibiotic use in the previous three months and patients with no history of periodontal treatment in last six months were recruited from the outpatient department of The Oxford Dental College, Hospital and Research centre, Bangalore. Subjects with oral pathologic conditions other than periodontal disease, pregnant and lactating women, chronic alcoholics and patients taking medications like corticosteroids, anti-inflammatory drugs, chemotherapeutic agents, anticancer drugs and immune modulators, which can alter the salivary cystatin C levels were excluded from the study.

A complete periodontal examination was performed, including recording of patient's chief complaint, the medical and dental history, an intra-oral examination & full-mouth periodontal probing. Clinical assessment was made using plaque index, gingival index, and gingival bleeding index, probing pocket depth, clinical attachment loss & bone loss. The subjects were classified into four groups viz., healthy controls, chronic periodontitis-smokers, chronic periodontitis-non smokers & aggressive periodontitis based on their periodontal status.

Recording of all clinical parameters was completed one day before the collection of samples. At the second visit, subjects were refrained from food or drink for a minimum of 30 minutes prior to the collection of saliva samples to avoid carryover effect from previous saliva stimulation.¹² Also, smoking was not permitted during the last 1hr prior to the saliva sample collection to avoid transient effect of cigarette smoking on salivary secretion.¹³ Saliva samples were collected from each subject prior to the therapy between noon and 2 p.m., to avoid the diurnal variations in the protein levels.¹² Navazesh's cotton roll method¹⁴ was used to collect saliva samples. Subjects were asked to swallow and three pre-weighed sterile cotton rolls were placed in mouth for minimum 10 minutes to collect unstimulated saliva from each subject. Samples of saliva contaminated by blood were discarded. At the end of collection, 2ml saliva was squeezed out of the wet cotton with the help of disposable plastic syringe into a labeled ice-cooled, sterile glass vial.

Samples were then stored at -20° C at St John's Medical College, Department of Biochemistry, Bangalore until biochemical assay was carried out. Samples were thawed from -20° C to a room temperature & centrifuged at 7000 - 8000 RPM for 20 minutes to remove the particulate matter and bacteria. After centrifugation, the clear supernatant designated as Clarified Human Whole Saliva (CHWS) was obtained by pipetting. Clarified Human Whole Saliva samples were then analyzed for the levels of cystatin C by using the Quantitative Sandwich Enzyme Immunoassay technique.

RESULTS

The clinical parameters such as plaque index, gingival index & gingival bleeding index were assessed for all the subjects in group I, while patients in group II, III & IV were evaluated for additional clinical parameters like probing pocket depth, loss of attachment. The salivary cystatin C (ng/ml) level for each group is presented in table 1. The levels were reported to decline in an order from group I > II > III > IV. When comparison was done for salivary cystatin C (ng/ml) levels between all four groups, pair wise *p* value (*p*=0.009) was found to be statistically significant only between group I & IV, and group II & IV. Although mean salivary cystatin C (ng/ml) value in group II (113.89±18.99) was greater when compared to group III (99.32 ±29.22), it was not statistically significant (*p* = 0.403).

Pearson's correlation of clinical variables with salivary cystatin C (ng/ml) was performed for all the groups and has been presented in table 2. In group I and II, plaque index & salivary cystatin C (ng/ml) levels were found to have positive correlation coefficient "r" of 0.396 & 0.370 and negative correlation coefficient "r" of -0.153 & -0.174 in group III and IV respectively. Gingival index & salivary cystatin C (ng/ml) levels exhibited positive correlation coefficient "r" of 0.428 & 0.154 in group II and III. However a negative correlation was found for group IV with a correlation coefficient "r" of -0.173. Salivary cystatin C (ng/ml) levels showed positive correlation with gingival bleeding index in group II & III with correlation coefficient "r" of 0.144 & 0.105, while it was found to have negative correlation coefficient "r" of -0.054 in group IV.

In all periodontitis groups (groups II, III & IV), mean attachment loss (mm) & salivary cystatin C (ng/ml) levels exhibited negative correlation coefficient "r" of -0.184, and -0.187, -0.004 respectively. Similar to the attachment loss, mean pocket depth (mm) & salivary cystatin C (ng/ml) levels also showed negative correlation coefficient "r" of -0.215 in group II, -0.477 in group III & -0.074 in group IV. Pair wise comparison of ROC curve analysis was performed between all the groups to find the discriminating potential (diagnostic values) of salivary cystatin C (ng/ml) based on Area under Curve (AUC) and has been presented in Table 3. When compared with group I (healthy controls), the discriminating potential of salivary Cystatin C (ng/ml) was good with AUC 0.07911 for group IV, while it had poor discriminating potential for group II & III with AUC 0.5467 & 0.6889.

DISCUSSIONS

Plaque-induced periodontal diseases are mixed infections associated with relatively specific groups of indigenous oral bacteria. Inflammation and bone loss are hallmarks of periodontal diseases which are mainly associated with proteolytic events brought about by enzymes such as cathepsins derived from both host as well as microorganisms. Under physiological conditions, the proteolytic activity of these enzymes is tightly controlled at several levels, and disturbance of its regulation instigates uncontrolled release of proteinases that exacerbate the chronic inflammatory condition, resulting in autolysis and tissue destruction seen in periodontitis.¹⁵ Saliva plays a protective role in maintaining oral health. It contains a broad array of antibacterial peptides with diverse functions.^{5, 8} Role of salivary cystatins, a family of low molecular weight antimicrobial peptides in modulating inflammatory response associated with periodontitis is well documented.¹⁶

The potential value of salivary cystatin C, a family 2 cysteine proteinase inhibitor in the pathogenesis of periodontal diseases has been extensively studied by many authors, markedly by Baron et al.¹¹ and Hensken et al.¹⁷ Based on their findings the present study was designed to analyze the levels of salivary cystatin C in periodontally healthy subjects, smokers with chronic periodontitis, chronic periodontitis non-smokers and aggressive periodontitis patients and to compare the difference in the levels of this antimicrobial protein in the selected study groups along with clinical parameters viz. plaque index, gingival index, gingival bleeding index, probing pocket depth and loss of attachment.

The present study reported highest levels of salivary cystatin C (ng/ml) in periodontally healthy subjects, ranging from 60.37 (ng/ml) to 146.35 (ng/ml) with mean value 114.37 ± 27.95 (ng/ml) when compared to periodontitis patients, which was in accordance with the study by Ito et al.¹⁰ and Baron et al.¹¹ This may be due to increased cathepsin activity associated with advanced periodontal inflammation, leading to the "proteolytic burst" which inactivated this proteinase inhibitor in periodontitis. The results in this study demonstrated greater amount of salivary cystatin C (ng/ml) values in smokers with chronic periodontitis (113.89 ± 18.99) when compared to non-smokers with chronic periodontitis (99.32 ± 29.22) which is quite similar to the study by Warfel et al.¹⁸, who reported that alveolar macrophages from smokers secreted more cystatin C in in-vitro cultures than such cells from non-smokers. This could be due to tobacco byproducts present within the smoke that served as an irritant, increasing constitutive secretion of cystatin C elaborated by alveolar macrophages.

While no evidence has so far been provided, it is important to note that patients with aggressive periodontitis exhibited least levels of salivary cystatin C (83.45 ± 24.26) in the present study. The decreased output of salivary cystatin C (ng/ml) in aggressive periodontitis cases as compared to chronic periodontitis could be attributed to several immuno-inflammatory factors involved in the pathogenesis of aggressive periodontitis. Leung-Tack et al.¹⁹ reported that cystatin C has a regulatory role in inflammatory process by down regulation of phagocytosis associated respiratory burst reaction displayed by polymorphonuclear neutrophils and by down regulation of their chemotactic activity. Thus, suggesting a potential role for cystatin C as a modulator of inflammation.

According to the findings in present study, markedly lower levels of salivary cystatin C (ng/ml) in aggressive periodontitis can further provide a clue to the recent citations of hyperresponsive nature of neutrophils that offset its physiological inhibitory activity against cysteine proteinases derived from *P. gingivalis* and *A. actinomycetamcomitans*.²⁰ This in turn could initiate rapid changes in the transcription rates of cathepsins and cystatin C, modifying the molecular pathways that lead to imbalance.

Furthermore, the present study also revealed positive and statistically significant correlation between salivary cystatin C levels and two clinical parameters i.e. gingival index and bleeding index in group II (chronic periodontitis smokers) and III (chronic periodontitis non-smokers), which was well in line with the previous studies by Hensken et al.¹⁷ This suggests that salivary cystatin C may serve as a probable biomarker of severity and chronicity of inflammation of the periodontium. When other clinical parameters were evaluated, salivary cystatin C (ng/ml) levels exhibited negative correlation with mean probing pocket depth and mean attachment loss in all periodontitis groups (group II, III and IV), suggestive of reduced protection against periodontal tissue breakdown and increasing disease severity with the low levels of cystatin C (ng/ml) present in saliva. This finding corroborates with the study by Lahet al²¹, who reported that the concentration of cystatin C in gingival tissue samples obtained from patients at various stages of periodontitis was significantly decreased when taken from sites with increased probing depths. A noteworthy finding of this study is lower levels of salivary cystatin C in chronic and aggressive periodontitis when compared to healthy controls. This down-regulation of salivary cystatin C production in periodontitis may be due to tissue pathology attributable to the persistence of unopposed proteolysis, which indicates that ascertaining the amount of this antibacterial protein in saliva may serve as a useful tool for monitoring the inflammatory periodontal disease status and its progression.

CONCLUSIONS

Within the limits of this study it can be concluded that cystatin C in saliva can be considered as a potent inflammatory biomarker to assess periodontal disease severity and its markedly low levels in aggressive periodontitis patients suggest that it is a fairly significant diagnostic biomarker and has a potential application as a screening tool for aggressive periodontitis. However further interventional as well as longitudinal studies are necessary to validate substantial changes in salivary cystatin C levels in assessing periodontal disease severity and monitor its progression as well as to determine the therapeutic significance of cystatin C as an antibacterial agent that could not be degraded in the proteolytic environment associated with periodontal disease.

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APPENDICES

Table 1: Comparison of Cystatin C (ng/ml) between Four Groups

Comparison Groups	Cystatin C (ng/ml)	
	Min-Max	Mean $\pm$ SD
Group I	60.37-146.35	114.37 $\pm$ 27.95
Group II	90.32-148.71	113.89 $\pm$ 18.99
Group III	42.54-142.17	99.32 $\pm$ 29.22
Group IV	37.51-127.60	83.45 $\pm$ 24.26
P value -ANOVA	F=4.936; P=0.004**	
Pair Wise P Value	Delta	P Value
Group I-Group II	0.24	1.000
Group I-Group III	14.82	0.389
Group I-Group IV	30.67	0.009**
Group II-Group III	14.58	0.403
Group II-Group IV	30.43	0.009**
Group III-Group IV	15.86	0.329

Table 2: Pearson Correlation of Clinical Variables with Cystatin C (ng/ml)

Comparison Groups	Pearson Correlation				
	Plaque Index vs Cystatin C	Gingival Index vs Cystatin C	Gingival Bleeding Index vs Cystatin C	Mean Attachment loss vs Cystatin C	Mean Pocket depth vs Cystatin C
Group I	0.396 (0.144)	-0.093 (0.743)	-0.331 (0.228)	-	0.030 (0.914)
Group II	0.370 (0.175)	0.428 (0.111)	0.144 (0.610)	-0.184 (0.512)	-0.215 (0.441)
Group III	-0.153 (0.586)	0.154 (0.584)	0.105 (0.710)	-0.187 (0.504)	-0.477 (0.072)
Group IV	-0.174 (0.534)	-0.173 (0.536)	-0.054 (0.850)	-0.004 (0.989)	-0.074 (0.793)

Table 3: ROC Curve Analysis for Salivary Cystatin C (ng/ml)



